Clinical Investigation / Araştırma

DOI: 10.4274/tjod.galenos.2024.84031

Turk J Obstet Gynecol 2024;21:142-52

True empty follicle syndrome is a subtype of oocyte maturation abnormalities

Gerçek boş folikül sendromu oosit olgunlaşma anomalilerinin bir alt türüdür

Şafak Hatırnaz[1], Ebru Hatırnaz[1], Justin Tan[2], Samettin Çelik[3], Canan Soyer Çalışkan[3], Alper Başbuğ[4], Gerçek Aydın[5], Ali Bahadırlı[6], Mehmet Bülbül[7], Handan Çelik[8], Aşkı Ellibeş Kaya[9], Nur Dokuzeylül Güngör[10], Seang Lin Tan[11,12], Mingju Cao[11], Michael H. Dahan[11,12], Sebati Sinan Ürkmez[13]

[1]*Mediliv Medical Center, Samsun, Turkey*
[2]*Toronto University Faculty of Medicine, Department of Obstetrics and Gynecology, Ontario, Canada*
[3]*Samsun Training and Research Hospital, Clinic of Obstetrics and Gynecology, Samsun, Turkey*
[4]*Düzce University Faculty of Medicine, Department of Obstetrics and Gynecology, Düzce, Turkey*
[5]*Eurofertil IVF Center, Bursa, Turkey*
[6]*Medicabil Hospital, Clinic of Obstetrics and Gynecology, Bursa, Turkey*
[7]*Karabük University Faculty of Medicine, Department of Obstetrics and Gynecology, Karabük, Turkey*
[8]*Ondokuz Mayıs University Faculty of Medicine, Department of Obstetrics and Gynecology, Samsun, Turkey*
[9]*Private Office, Obstetrics and Gynecology, Samsun, Turkey*
[10]*Bahçeşehir University Medical Park Göztepe Hospital, Clinic of Obstetrics and Gynecology, İstanbul, Turkey*
[11]*OriginElle Fertility Clinic and Women's Health Centre, Montreal, Quebec, Canada*
[12]*McGill University Faculty of Medicine, Department of Obstetrics and Gynecology, Quebec, Canada*
[13]*Ondokuz Mayıs University Faculty of Medicine, Department of Medical Biochemistry, Samsun, Turkey*

Abstract

Objective: To review the outcomes of in vitro maturation (IVM) and in vitro fertilization (IVF) in women with empty follicle syndrome (EFS). The study evaluated the genetic underpinnings of EFS by analyzing mutations.

Materials and Methods: This retrospective case series involving 17 women with EFS over at least 2 IVF cycles was conducted. The study also employed whole-exome sequencing to analyze the genetic mutations. The treatment approaches included letrozole-primed IVM, follicle-stimulating hormone (FSH)-human chorionic gonadotrophin (hCG)-primed IVM, and conventional IVF.

Results: The average female age was 31.5±4.6 years, and the duration of infertility was 7.3±3.5 years. Four patients underwent IVF. IVM oocyte collections yielded oocytes in 12 of 13 subjects. Of these, 75% (9/12) yielded MII oocytes after 48 h of IVM media incubation. Six subjects had fertilized embryos, resulting in a 40.9% intracytoplasmic sperm injection (ICSI) fertilization rate (9 embryos/22 MII oocytes). Genetic analysis revealed mutations in seven patients. This study demonstrated the partial efficacy of letrozole-primed IVM plus growth hormone and FSH-hCG primed IVM protocols. No pregnancies or live births were recorded after IVM. One ongoing pregnancy post-IVF and one spontaneous live birth were observed.

Conclusion: Inter-cycle variabilities were observed in women with oocyte maturation abnormalities (OMAs). Almost all patients with EFS had oocytes collected during IVM following IVF. These oocytes have limited potential for maturation, fertilization, and live birth, as demonstrated by the low rates observed after IVM culture and ICSI. These conditions are observed in OMAs due to defects in the oocyte machinery. The proposed flowchart provides a comprehensive classification approach for various forms of EFS.

Keywords: Empty follicle syndrome, in vitro maturation, oocyte maturation abnormalities, oocyte maturation arrest

PRECIS: Empty follicle syndrome.

Address for Correspondence/Yazışma Adresi: Assoc. Prof. Nur Dokuzeylül Güngör,
Bahçeşehir University Medical Park Göztepe Hospital, Clinic of Obstetrics and Gynecology, İstanbul, Turkey
Phone: +90 532 383 49 65 **E-mail:** drnur9eylul@hotmail.com **ORCID ID:** orcid.org/0000-0001-8859-0639
Received/Geliş Tarihi: 27.05.2024 **Accepted/Kabul Tarihi:** 16.07.2024

Öz

Amaç: Bu çalışmada, boş folikül sendromu (BFS) olan kadınlarda in vitro matürasyon (IVM) ve in vitro fertilizasyon (IVF) tedavi sonuçlarını gözden geçirmeyi amaçladık. Çalışmada, BFS'nin genetik temelleri analiz edilerek mutasyonlar değerlendirildi.

Gereç ve Yöntemler: En az 2 IVF döngüsünde BFS olan 17 kadını içeren retrospektif bir olgu serisi gerçekleştirildi. Genetik mutasyonları analiz etmek için bütün ekzon dizilimi kullanıldı. Tedavi yaklaşımı, letrozol ile ön hazırlıklı IVM, folikül uyarıcı hormon (FSH)-insan koryonik gonadotropini (hCG) ile ön hazırlıklı IVM ve konvansiyonel IVF'yi içeriyordu.

Bulgular: Ortalama kadın yaşı 31,5±4,6 yıl ve infertilite süresi 7,3±3,5 yıl idi. Dört hastada IVF uygulandı. IVM ile, 13 olgunun 12'sinde oosit elde edildi. Bu toplamaların %75'inde (9/12) IVM medyumunda 48 saat inkübasyon sonrası MII oositler elde edildi. Altı hastada embriyo elde edildi ve fertilizasyon oranı %40,9 (9 embriyo/22 MII oosit) gözlendi. Genetik analiz ile yedi olguda mutasyon saptandı. Çalışma, letrozol ile ön hazırlıklı IVM artı büyüme hormonu ve FSH-hCG ile ön hazırlıklı IVM protokollerinin kısmi etkinliğini gösterdi. IVM sonrası hamilelik ve canlı doğum kaydedilmedi. IVF sonrası devam eden bir gebelik ve bir spontan canlı doğum gözlendi.

Sonuç: Oosit matürasyon anormallikleri (OMA) olan kadınlarda döngüler arası değişkenlikler gözlendi. IVF sonrası IVM sırasında BFS'li hastaların neredeyse tamamında oositler toplandı. Bu oositler, IVM kültürü ve intrasitoplazmik sperm enjeksiyonu sonrası düşük oranlarla gösterildiği üzere, sınırlı matürasyon, döllenme ve canlı doğum potansiyeline sahiptir. Bunlar, oosit makinelerindeki kusurlar nedeniyle OMA'da görülen durumlardır. Önerilen akış şeması, BFS'nin çeşitli formlarının sınıflandırılmasına yönelik kapsamlı bir yaklaşım sunmaktadır.

Anahtar Kelimeler: Boş folikül sendromu, in vitro matürasyon, oosit matürasyon anormallikleri, oosit matürasyon arresti

Introduction

Failure to yield oocytes from fully developed follicles at oocyte pick up (OPU) 35-37 hours after human chorionic gonadotropin (hCG) injection during an in vitro fertilization (IVF) cycle is defined as empty follicle syndrome (EFS)[1]. EFS has been a subject of intense debate regarding whether it is a true entity or failure of IVF cycle management. Before the implementation of gonadotropin-releasing hormone analogs (GnRHa) for triggering in antagonist IVF cycles, EFS was subdivided into two subtypes: The first is known as false-EFS (fEFS), which is due to the lack of hCG application before OPU or low efficacy of applied hCG; the second is referred to as genuine-EFS (gEFS), which is not related to errors in hCG but a repeated failure in at least two consecutive cycles to collect oocytes despite adequate dose and timing of hCG application[2].

The prevalence of gEFS ranges from 0.045% to 7% in the literature[3-6]. Hourvitz et al.[7] published the first successful pregnancies and live births via in vitro maturation (IVM) in women with gEFS, the cause of which was accepted as part of the oocyte maturation abnormalities (OMAS). Human genetic studies have revealed that mutations related to oocyte maturation and zona pellucida (ZP) play crucial roles in oocyte development[8]. Most cases of EFS were found to have mutations related to ZP proteins[9-11]. To date, more than 25 mutations in ZP-related proteins have been reported[12].

For follicular growth and oocyte developmental competence, the corona radiata projections and oocyte microvilli must be in contact with the ZP. Absence of, or deficiency of ZP formation impairs bidirectional communication between the oocyte and surrounding cumulus cells and diminishes oocyte development by destabilizing the cumulus, and affected subjects may develop female infertility[13-15]. Defects in ZP, especially those related to integrity rather than thickness, result in a lack of oocyte development, defective folliculogenesis, and early granulosa cell apoptosis, which may explain the pathophysiology of EFS[16]. Hatirnaz et al.[17] previously reported intracycle and intercycle variabilities in women with OMAs[17]. Among patients who had undergone 2-8 previous IVF attempts, some experienced EFS in at least two cycles and experienced oocyte maturation arrest and biochemical pregnancies in different cycles. This observation suggests that the follicles are not empty in women diagnosed with gEFS, but rather are due to oocyte failure. It is noteworthy that even in women with gEFS associated with genetic mutations, there may be potential for improvement in oocyte yield and maturation. EFS can be redefined as 1) EFS as part of OMAs, 2) Genuine EFS (gEFS) (altered gene expressions, or early apoptosis, etc.), 3) GnRHa use-related EFS, and 4) hCG-related EFS (fEFS). FEFS is a poorly researched condition that affects some women undergoing IVF treatment. HCG-treatment-related errors appear to be the main mechanism underlying fEFS. Improper hCG administration has often been the most common cause[3]. Some studies have suggested that increasing the dose of gonadotropins, administering a second (rescue) dose of hCG, or extending the duration from hCG injection to oocyte collection may improve the chances of retrieving oocytes.

The effectiveness of these approaches remains unclear and requires further investigation[18]. A potential preventative measure of serum hCG level the day after the trigger and a second bolus of hCG administration has been proposed to prevent against fEFS[5]. Clinicians can trigger GnRHa, especially in hyperresponder patients, because of the shorter surge effect of GnRHa compared with urinary or recombinant hCG[19-21]. The shorter duration of luteinizing hormone (LH) activity observed in GnRHa cells substantially limits the risk of OHSS but may also contribute to diminished oocyte performance, even leading to false EFS[22]. The mechanism of fEFS in this case was different from that observed with hCG, where GnRH agonists induce the release of LH and follicle-stimulating hormone (FSH) from the pituitary. Therefore, with any temporary or permanent factor leading to the dysfunction of the pituitary gland (i.e., hypogonadotropic-hypogonadism), the expected flare action of the analog can be altered and may be responsible for diminished

oocyte yield, maturation problems, and EFS[23]. For cases with a history of previous EFS, a dual trigger can be planned. During GnRH agonist-triggered cases, especially for patients with the predisposing factors listed subsequently, if no oocyte is yielded from an ovary, the procedure can be stopped, and a second trigger is planned, usually consisting of hCG. Here we performed a case series, identified 17 women with gEFS who had letrozole-primed IVM plus growth hormone (GH), FSH-hCG-primed IVM treatment, and IVF, and investigated oocyte maturation and embryo development, as well as pregnancy outcomes in women who had repetitive EFS after undergoing at least two IVF cycles. Additionally, in this paper, we introduce a flowchart for managing the multiple forms of gEFS. This study aimed to review IVM and IVF treatment outcomes in women with EFS and evaluate the genetic underpinnings of EFS by analyzing mutations.

Materials and Methods

In this case series study, 17 women who had gEFS in their previous (at least two) IVF cycles were detected. Only patients who had gEFS after hCG trigger were included in the study group. FEFS and EFS secondary to GnRH agonist triggering were not included in the study. Demographic characteristics and basal hormonal parameters were all evaluated and recorded. The 17 women were contacted, and written informed consent was obtained from the women studied. This study was approved by the Clinical Research Ethics Committee of Samsun University, Turkey (decision date: 26.04.2023, decision number: 2023/8/20).

Letrozole-priming IVM Plus Growth Hormone

Letrozole 2.5 mg tablets (PO) twice daily started on day 3 of the menstrual cycle for 5 days following the initial transvaginal ultrasound examination. On day 7, a second transvaginal ultrasound examination was performed to measure follicular growth and endometrial thickness. Recombinant GH (*Genotropin* 36 IU/12 mg) 2 mg/day subcutaneously was added to the treatment regimen on day 10 for 6 days. A third ultrasound examination was conducted to assess the antral follicles. Once at least 2-3 follicles reached a diameter of 10-12 mm and the endometrial thickness reached 8 mm, a 6.500 IU subcutaneous injection of hCG (*Ovitrelle, Merck Serono, Turkey*) was administered 38 h prior to oocyte collection. Oocyte retrieval was performed under an aspiration pressure of 80 mmHg using an 18-gauge aspiration needle with a double lumen for continuous flushing. Embryos obtained in this protocol were frozen either at the cleavage or blastocyst stages, and a frozen-thawed embryo transfer cycle was performed 1-2 months after the procedure.

FSH-hCG-priming IVM

Recombinant FSH (*Gonal-f, Serono*) was administered subcutaneously at a daily dose of 75-150 IU, starting on day 3 of the menstrual cycle and continuing for 3 days following the initial transvaginal ultrasound examination. On day 7, a second transvaginal ultrasound examination was performed to measure follicular growth and endometrial thickness. Around day 9-10, a third ultrasound examination was performed to measure the size of the antral follicles. When follicles reach 10-12 mm in diameter and the endometrial thickness reached 8 mm, recombinant hCG 6500 IU (*Ovitrelle, Merck Serono*) was administered 38 h before oocyte retrieval. Oocyte retrieval was performed with a 17-18-gauge aspiration needle with a double lumen. Embryos obtained in this protocol were transferred to the fresh cleavage stage, and the remaining embryos, if any, were frozen at the cleavage stage.

Luteal Phase Support

The luteal phase was supported with estradiol valerate at a dose of 6-8 mg per day until fetal heartbeat was detected during ultrasound examination. Progesterone 200 mg capsules (800 mg/day (*Koçak Farma, İstanbul, Turkey*) intravaginally were used routinely in both letrozole primed IVM plus GH and in FSH-hCG IVM cycles until the 12th week of gestation.

Laboratory Procedures

Laboratory procedures for IVM were performed using both methods according to a modified protocol, as previously described[24]. The authors have been using MediCult IVM culture systems (CooperSurgical, USA) since 2007. A SAGE Vitrification Kit (Cooper Surgical, USA) was used for the vitrification and warming procedures.

The maturation process was assessed at 26 and 48 h. Oocytes that had matured at 26 h underwent intracytoplasmic sperm injection (ICSI) as further culture was deemed unnecessary. Any remaining immature oocytes were maintained in IVM culture until 48 h. Oocytes that matured at 48 h were then subjected to ICSI. The cleavage-stage embryos were graded according to the system reported by Hsu et al.[25]. Blastocysts were graded according to a previously reported system by Neuber et al.[26].

A pregnancy test was administered on either day 10 post-blastocyst transfer or day 12 following cleavage-stage embryo transfer. Serum hCG levels exceeding 5 IU/L during the initial assessment indicated a positive pregnancy. Clinical pregnancy was defined as the detection of an intrauterine fetus with a heartbeat on ultrasound. Ongoing pregnancy was defined as the pregnancies over 12th weeks of gestation. A spontaneous abortion is defined as the presence of an empty gestational sac or a gestational sac containing an embryo or fetus without fetal heart activity within the first 12 weeks of gestation. Live birth was defined as the birth of a living child born after 28 gestational weeks. Falling beta hCG levels without visualization of pregnancy on ultrasound were considered as biochemical pregnancy loss.

Whole-exome Sequencing

Genetic mutations in 9 out of 17 patients with gEFS were analyzed by whole-exome sequencing (WES). Briefly, DNA

was extracted from whole blood samples using a QIAmp kit (Hilden, Germany) in a QIAcube HT system (Hilden, Germany) following the manufacturer's instructions (Richards et al. 2015). Library preparation/comparison was conducted using a QIAseq Index I set A kit (Hilden, Germany) in combination with a custom-designed targeted NGS panel (CDHS-15607Z-1008) for female infertility-associated genes.

Statistical Analysis

Statistical analyses were performed using SPSS software (IBM SPSS Statistics, Version 21.0, USA). Descriptive statistics, including means and standard deviations, were calculated to summarize the demographic and baseline clinical characteristics of the study participants.

Results

The average age of the 17 female participants was 31.5±4.6 years (range: 24-38), and the average duration of infertility averaged 7.3±3.5 years (range: 1.5-15.0). Basal serum measurements revealed FSH levels at 8.24±3.56 IU/L, LH levels at 8.93±6.40 IU/L, estradiol levels at 59.67±44.48 pg/mL, progesterone levels at 0.58±0.29 ng/mL, thyroid stimulating hormone levels at 1.93±0.99 mIU/mL, T3 levels at 2.96±0.40 pg/mL, T4 levels at 1.16±0.25 ng/mL, anti-Mullerian hormone levels at 2.96±2.03 ng/mL, PRL levels at 21.17±9.74 ng/mL, and antral follicle count of 17.4±8.9. Notably, four (out of the 17) patients did not undergo the IVM procedure. IVM procedures resulted in successful oocyte collection in 12 of 13 subjects. Among these, 9 out of 12 subjects (75%) yielded MII oocytes after 48 h of incubation with IVM medium (with a range of mature oocytes in those who matured being 2-8). Only 6 subjects ended up with fertilized embryos (9 of 22 MII oocytes total. This represents a fertilization rate of 40.9% with ICSI. None of the subjects in this study had experienced any IVM pregnancies. One patient achieved an ongoing pregnancy following an IVF cycle (Case 5), while another case (Case 2) resulted in a spontaneous live birth.

The detailed demographic, clinical, and laboratory characteristics of each patient are presented in Table 1.

The application of IVF and IVM treatments and outcomes are outlined in Table 2.

Wes analysis was performed on 9 subjects. Genetic mutations (FSHR mutations, TACR3 mutation, LHX4, mutation, STAG3 mutation, ZP1 mutation, ZP3 mutation, PATL2 mutation and LHCGR mutation) were detected in 7 women. Cases 7 and 8 had no mutation in WES analysis. In eight patients (Case 1, 3 9, 10, 14-17), WES analysis was not performed, as indicated in Table 3.

- In Case 2, a heterozygous mutation in STAG3 was identified on Exon 4, specifically at nucleotide position c.319G>A. This mutation follows an autosomal recessive (AR) inheritance pattern.
- Case 4 exhibited a homozygous mutation in the *LHCGR* gene located in Exon 11 at nucleotide position c.970_971del A>G, consistent with AR inheritance.
- Case 5 was found to have a novel homozygous mutation in the *ZP1* gene in Intron 11, denoted as c.1775.3C>G, which also followed an AR inheritance pattern.
- A more complex genetic profile was observed in Case 6, which exhibited three mutations: a homozygous mutation in the *FSHR* gene on Exon 10 (p. S680N), a heterozygous mutation in the same gene but at a different site (Exon 10, p. A307T), and additional heterozygous mutations in the *TACR3* gene (Exon 2, c.737c>t) and *ZP3* gene (Exon 2, c.382A>C). The FSHR and TACR3 mutations follow AR inheritance, whereas the ZP3 mutation is autosomal dominant.
- Case 11 showed a heterozygous mutation in LHX4 located at c.1158T>A, which followed an AR inheritance pattern.
- In Case 12, two homozygous mutations were detected in PATL2: one in Exon 7 (c.320C>T) and the other in Intron 7 (c.446+1G>C), both consistent with AR inheritance.
- Case 13 was identified with a homozygous mutation in ZP1 on Exon 12, which was noted as C1775-3C>A, following an AR pattern.

Detailed data are presented in Table 3.

Discussion

Our paper introduces several novel insights into the study of EFS. First, we present EFS as a subtype of OMAs that is determined by genetic mutations through a comparative study. Second, it explores the role of a letrozole-primed plus GH IVM protocol in women with gEFS. OMAs often result in the collection of 100% immature oocytes, even after triggering with hCG, GnRH agonist, or both[27,28]. IVM is performed to collect oocytes from follicles measuring 2-10 mm, smaller than the size in IVF cycles. IVM, being independent of the development of LH receptors in the follicle, as required in IVF, could serve as a treatment for EFS. The study investigated 17 women diagnosed with gEFS. Out of the 9 women analyzed by WES, seven had various mutations noted (FSHR mutations, TACR3 mutation, LHX4, mutation, STAG3 mutation, ZP1 mutation, ZP3 mutation, PATL2 mutation and LHCGR mutation). Two women did not present with mutations in their WES analysis. The results of this study indicated that IVM for gEFS, whether using the letrozole-primed IVM + GH protocol or FSH-hCG primed IVM, may result in oocytes and embryos in rare cases but is not likely to result in a live birth. This study also demonstrated for the first time that EFS is a subtype of OMA and that genetic testing for mutations is highly recommended if available.

Since its first report, EFS has been a hot topic of debate in the field of assisted reproductive technology[1]. EFS is classified as g EFS, in which repeated failure to retrieve oocytes with appropriate blood hCG levels is observed, and fEFS, in which a low blood level of hCG (<40 IU/L) is detected either due to misuse of hCG for triggering or low bioavailability of the HCG used[29,30]. Filtrates of follicular fluids collected from patients diagnosed with gEFS during a stimulated IVF cycle were investigated, and it was demonstrated that immature

Table 1. Demographic and clinical variables

Patients	Age (years)	Time to infertility (years)	Basal serum FSH (IU/L)	Basal serum LH (IU/L)	Basal serum E2 (pg/mL)	Basal serum P4 (ng/mL)	Basal serum TSH (mU/mL)	Basal serum T3 (pg/mL)	Basal serum T4 (ng/mL)	Basal serum AMH (ng/mL)	Basal serum PRL (ng/mL)	Total AFC (number)
Case 1	24	4	11.10	5.70	21.20	0.40	3.53	3.50	1.12	2.10	22.90	9
Case 2	29	2	11.20	2.53	125.00	0.20	1.10	3.60	0.90	0.43	18.70	8
Case 3	33	15	4.70	3.02	40.00	0.30	1.25	2.50	1.40	4.60	47.60	20
Case 4	37	11	18.40	27.2	38.00	0.30	1.98	2.85	1.00	0.50	21.70	5
Case 5	32	5	8.05	9.00	35.25	0.60	1.52	2.78	1.05	6.44	34.78	26
Case 6	31	7	10.70	6.50	10.00	0.45	2.31	3.45	1.24	1.04	24.10	6
Case 7	29	8	10.08	4.68	35.83	0.46	2.48	3.00	1.27	0.43	6.16	5
Case 8	29	1.5	7.35	6.40	68.56	0.28	3.19	2.89	1.24	4.60	12.68	32
Case 9	32	12	6.60	7.00	15.00	0.76	4.40	2.64	0.99	3.61	20.09	20
Case 10	35	6	6.45	20.61	53.10	0.36	2.06	2.86	0.93	5.78	24.14	18
Case 11	28	7	3.04	5.97	143.13	0.89	1.08	2.45	1.22	3.08	8.99	28
Case 12	25	7	8.46	7.80	62.01	0.97	1.05	2.45	0.89	0.97	17.50	22
Case 13	38	10	6.86	4.98	42.08	0.78	2.13	3.04	1.45	1.64	14.27	12
Case 14	37	10	8.79	12.40	49.32	0.97	1.05	3.04	0.96	5.45	19.12	28
Case 15	38	4	6.45	11.46	46.02	0.98	1.45	3.45	1.42	3.05	20.05	23
Case 16	25	7	8.46	11.56	62.01	0.97	1.05	2.45	0.89	4.98	17.05	22
Case 17	33	7	3.50	5.040	168.00	0.20	1.22	3.45	1.800	1.76	30.10	11
Total (mean ± SD)	31.5±4.6	7.3±3.5	8.24±3.56	8.93±6.40	59.67±44.48	0.58±0.29	1.93±0.99	2.96±0.40	1.16±0.25	2.96±2.04	21.17±9.74	17.35±8.89

AFC: Antral folicle count, AMH: Anti-Müllerian hormone, E2: Estradiol, FSH: Folicle stimulating hormone, hCG: Human chorionic gonadotropin, LH: Luteinizing hormone, P4: Progesterone, PRL: Prolactin, SD: Standard deviation, TSH: Thyroid stimulating hormone

Table 2. Treatments administered to patients and their outcomes

Patients	OMA diagnosis	IVF treatments			IVM treatments				Additional information
		IVF cycle numbers	Collected oocytes	Outcome	IVM cycle numbers	Collected oocytes	Matured oocytes	Outcome	
Case 1	EFS/OMA TYPE V	4	0, 0, 4, 0	1 Spontaneous abort (from a fresh transferred day 3 and grade 2 embryo), others no fertilization	3	4, 1, 4	3, 0, 2	Two ET (each fresh transferred day 3 and grade 2 embryos), negative hCG test	Severe OAT, spontaneous abortion, and EFS as part of OMA
Case 2	EFS/POI/ POF	2	0, 0	None	4	0, 5, 3, 3	0, 2, 0, 2	Two ET (each fresh transferred day 3 and grade 2 embryos), negative hCG test/ Frozen embryos	Three cases of spontaneous biochemical pregnancy loss, one case of spontaneous livebirth, EFS as part of OMAs
Case 3	EFS	3	0, 0, 0	None	2	2, 4	0, 0	None	True EFS
Case 4	EFS/POI/ POF	3	0, 0, 0	None	2	2, 5	2, 0	One ET (fresh transferred day 3 and grade 2 embryo), negative hCG test	True EFS
Case 5	EFS/Zona free	4	0, 0, 1, 2	3rd attempt: Zone-free oocyte ICSI and embryo frozen (grade 2). 4th attempt: 2 grade 2 embryos. One transferred frozen, pregnancy	No IVM	-	-	-	EFS as part of the OMAs, oocytes with zona free formation were also obtained.
Case 6	EFS/POI/ POF	4	0, 0, 0, 1	Degenerated oocyte	2	1, 1	0, 0	None	True EFS and zona-free degenerated
Case 7	EFS/POI/ POF	4	0, 0, 0	None	1	0	0	None	True EFS
Case 8	EFS	2	0, 0	None	1	7	3	One ET (frozen/thawed transferred day 3 and grade 2 embryo), negative hCG test	True EFS
Case 9	EFS	2	0, 0	None	No IVM	-	-	-	True EFS
Case 10	EFS	3	0, 0, 0	None	1	3	0	None	True EFS

Table 2. Continued

Patients	OMA diagnosis	IVF treatments			IVM treatments				Additional information
		IVF cycle numbers	Collected oocytes	Outcome	IVM cycle numbers	Collected oocytes	Matured oocytes	Outcome	
Case 11	EFS	2	0, 0	None	1	6	3	One ET (fresh transferred day 3 and grade 2 embryo), negative hCG test	Spontaneous biochemical pregnancy loss and EFS as OMAs
Case 12	EFS	5	0, 0, 0, 0	None	No IVM	-	-	-	EFS as an OMA component One of three sisters with ZP1 mutation in a consanguineous family
Case 13	EFS	3	0, 0, 0	None	No IVM	-	-	-	EFS as an OMA component
Case 14	EFS	2	0, 0	None	1	12	0	None	Male azoospermia, true EFS
Case 15	EFS	2	0, 0	None	1	14	0	None	True EFS
Case 16	EFS	2	0, 0	None	1	8	0	None	Ture EFS
Case 17	EFS/OMA TYPE V	3	0, 0, 1	Biochemical pregnancy loss (from a fresh transferred day 3 and grade 2 embryo)	2	5, 2	5, 0	Two ET (each fresh transferred day 3 and grade 2 embryos), negative hCG test	EFS as an OMA component

EFS: Empty folicle syndrome, ET: Embryo transfer, hCG: Human chorionic gonadotropin, IVF: In vitro fertilization, IVM: In vitro maturation, OAT: Oligoasthenoteratozoospermia, OMA: Oocyte maturation arrest, OMAs: Oocyte maturation abnormalities, POF: Premature ovarian failure, POI: Premature ovarian insufficiency, ZP1: Zona pellucida glycoprotein 1

oocytes were present in the filtrates and that these could mature *in vitro* using IVM culture media[31]. This finding, though it is only a case report, showed that the follicles were not empty. Inan et al.[32] reported the whole gene expression profile of granulosa cells from the follicular fluid of a woman who underwent three repeated gEFS and showed that around 160 genes were differentially expressed. This study is the first to demonstrate the role of gene expressions that trigger early apoptosis in healthy oocytes and fail maturation. Thin ZP was associated with early apoptosis of oocytes present in 200 preantral follicles. Ovarian aging or low ovarian reserve is also a cause of gEFS[32,33]. A possible genetic cause of gEFS was reported by Onalan et al.[34], in which two sisters with moderate sensorineural deafness were diagnosed with gEFS due to alterations in the transient and sequential expression of epidermal growth factor, which is essential in the meiotic resumption process. The recurrence rate of EFS was reported to be 15.8%[35]. Baum et al.[35] found that patients with recurrent EFS exhibited significantly prolonged infertility and lower estrogen levels compared with those with sporadic EFS. However, their study did not include hCG hormone analysis; thus, the differentiation of EFS subtypes was not performed. Additionally, no genetic screening was performed for either sporadic or recurrent cases. Therefore, elucidating the underlying cause is challenging based on these data.

Mutation studies related to female infertility have revealed the involvement of ZP1-4, LHCGR, STAG3, and mutations related to premature ovarian failure (POF) that play a role in the pathogenesis of EFS. Thus, EFS, POF, and resistant ovary syndrome were included as OMA subtypes.

The significance of EFS in relation to mutations in the *LHCGR* gene[36,37]. We identified an LHCGR mutation in one of our EFS cases (Case 4) in which IVM resulted in oocyte retrieval and embryo transfer, but pregnancy was not achieved.

Furthermore, the relevance of EFS has been emphasized in connection with mutations in the *ZP1* gene[38,39]. In our study, a noteworthy observation was made regarding the ZP1 mutation within a consanguineous family, specifically involving three sisters. It exhibited distinct manifestations in the three sisters, particularly in the eldest sister (Case

Table 3. Mutation screening in patients

Patients	Mutation 1 (zygosity/ inheritance)	Gene 1	Nucleotide 1	Mutation 2 (zygosity/ inheritance)	Gene 2	Nucleotide 2	Mutation 3 (zygosity/ inheritance)	Gene 3	Nucleotide 3	Mutation 4 (zygosity/ inheritance)	Gene 4	Nucleotide 4
Case 1	Not evaluated	-	-	-	-	-	-	-	-	-	-	-
Case 2	STAG3 (Het./AR)	Exon 4	c.319G>A	-	-	-	-	-	-	-	-	-
Case 3	Not evaluated	-	-	-	-	-	-	-	-	-	-	-
Case 4	LHCGR (Hom./AR)	Exon 11	c.970_971 DEL A>G	-	-	-	-	-	-	-	-	-
Case 5	ZP1 (Hom./AR)	Intron 11	c.1775.3C>G (NOVEL)	-	-	-	-	-	-	-	-	-
Case 6	ZP3 (Het./AD)	Exon 10	p. S680N	FSHR (Het./AR)	Exon 10	p. A307T	TACR3 (Het./AR)	Exon 2	c.737c>t	FSHR (Hom./AR)	Exon 2	c.382A>C
Case 7	No mutation was detected	-	-	-	-	-	-	-	-	-	-	-
Case 8	No mutation was detected	-	-	-	-	-	-	-	-	-	-	-
Case 9	Not evaluated	-	-	-	-	-	-	-	-	-	-	-
Case 10	Not evaluated	-	-	-	-	-	-	-	-	-	-	-
Case 11	LHX4 (Het./AR)	Exon 6	c.1158T>A	-	-	-	-	-	-	-	-	-
Case 12	PATL2 (Hom./AR)	Exon 7	c.320C>T	PATL2 (Hom./AR)	Intron 7	c.446+1G>C	-	-	-	-	-	-
Case 13	ZP1 (Hom./AR)	Exon 12	c.1775-3C>A	-	-	-	-	-	-	-	-	-
Case 14	Not evaluated	-	-	-	-	-	-	-	-	-	-	-
Case 15	Not evaluated	-	-	-	-	-	-	-	-	-	-	-
Case 16	Not evaluated	-	-	-	-	-	-	-	-	-	-	-
Case 17	Not evaluated	-	-	-	-	-	-	-	-	-	-	-

FSHR: Follicle stimulating hormone receptor, LHCGR: Luteinizing hormone/chooriogonadotropin receptor, LHX4: LIM homeobox 4, PATL2: PAT1-like protein 2, STAG3: Stromal antigen 3, TACR3: Tachykinin receptor 3, ZP1: Zona pellucida glycoprotein 1, ZP3: Zona pellucida glycoprotein 3

12), which was included in our study. These manifestations included EFS, oocyte maturation arrest, and the production of mature oocytes with successful embryo transfer in one cycle. Additionally, her IVF cycles produced zona-free oocytes. The genetic analysis of this case was performed at the outer center. Although we have a report in our archives indicating the presence of a ZP1 mutation, we do not have the details. Therefore, it could not be included in Table 3. The genetic profile of the family has been published[40]. Additionally, in our WES analysis, we identified two distinct PATL2 mutations in this case. We also observed patients with gEFS during IVF cycles while having spontaneous biochemical pregnancies and sometimes mature oocytes during other IVF cycles. This finding is novel, and it is difficult to determine whether these patients have EFS or not, but we can accept these cases as subtypes of OMAs.

According to our WES analysis, two distinct mutations in ZP1 were identified in cases 5 and 13. The mutation detected in Case 5 is novel; however, it is located within Intron 11 of the *ZP1* gene, suggesting no phenotypic impact. Case 5 was diagnosed with EFS as part of OMAs. In the initial two IVF attempts, no oocytes were obtained. In the third IVF attempt, only one zona-free oocyte was retrieved, and ICSI was performed, resulting in embryo freezing. In the fourth IVF attempt, two normal oocytes were retrieved. Successful ongoing pregnancy was achieved through embryo transfer. Case 13, who was also diagnosed with EFS as part of OMAs, experienced this condition in two IVF attempts. In another IVF attempt, a germinal vesicle arrest. Both cases did not receive IVM treatment.

Moreover, the emphasis on EFS has been placed in the context of mutations in the *ZP3* gene[41,42]. Within our study, Case 6 was diagnosed with premature ovarian ınsufficiency (POI) and true EFS. Additionally, zona-free oocytes were observed in some cycles. Intriguingly, based on WES analysis, one mutation in ZP3 and two mutations in FSHR. The patient underwent four IVF attempts, with oocytes failing to be retrieved in three of the cases. In the fourth attempt, an oocyte was obtained, but it degenerated. Two attempts of IVM resulted in the retrieval of one oocyte each time; however, subsequent observations revealed zona-free oocytes and subsequent degeneration in the germinal vesicle stage.

In one of our cases (Case 2) with g-EFS in her previous two consecutive IVF cycles, we determined a STAG3 mutation in her WES analysis (this mutation was previously a variant of uncertain significance but likely benign according to Association for Clinical Genomic Sciences classification). She had a history of biochemical pregnancy prior to IVF attempts and was diagnosed with POI. We performed IVM cycles, obtained embryos, and transferred them twice but failed to achieve pregnancy. However, the patient had three consecutive spontaneous biochemical pregnancies while waiting for another frozen-thawed embryo transfer. The last result resulted in the delivery of a healthy baby. The clinical course of the patient was interesting, beginning with POI and EFS and ending with spontaneous pregnancy.

When we looked at the cases studied, apart from the FSH-hCG primed IVM group, in which all cases were gEFS, the remaining nine women treated with letrozole-primed IVM + GH had intercycle variabilities either in their previous IVF cycles or in their IVM cycles. This finding is important and explains why EFS has follicles that are not empty, providing new evidence that EFS is a subtype of OMAs[17,27].

We propose a novel flowchart for the clinical management of all types of EFS (Figure 1). Apart from gEFS, Functional EFS, and GnRH-related EFS, we describe a novel subtype: EFS as a subtype of OMAs. In managing this entity, repeating the cycle for accurate diagnosis or directly investigating mutations via WES may be recommended. Because of the importance of intercycle variability, mutation analysis before a second attempt is preferable.

Study Limitations

Our study has some limitations. The small sample size and retrospective design may limit the generalizability of our findings. Therefore, studies with larger series should be scheduled to draw proper conclusions regarding the use of letrozole IVM plus GH therapy.

Conclusion

Almost all patients diagnosed with EFS after IVF had oocytes collected during IVM, indicating that the follicles were not truly empty. These oocytes have limited potential for maturation and fertilization, as evidenced by the low success rates following IVM culture and ICSI. The conditions observed in these OMAs stem from defects in the oocyte machinery, resulting in oocyte and cytoplasmic immaturity. Despite these challenges, pregnancies did occur in some subjects, suggesting that EFS can occasionally be overcome. The current evidence does not support the success of IVM in patients with g-EFS; however, the number of cases studied is limited, and further research is needed.

Ethics

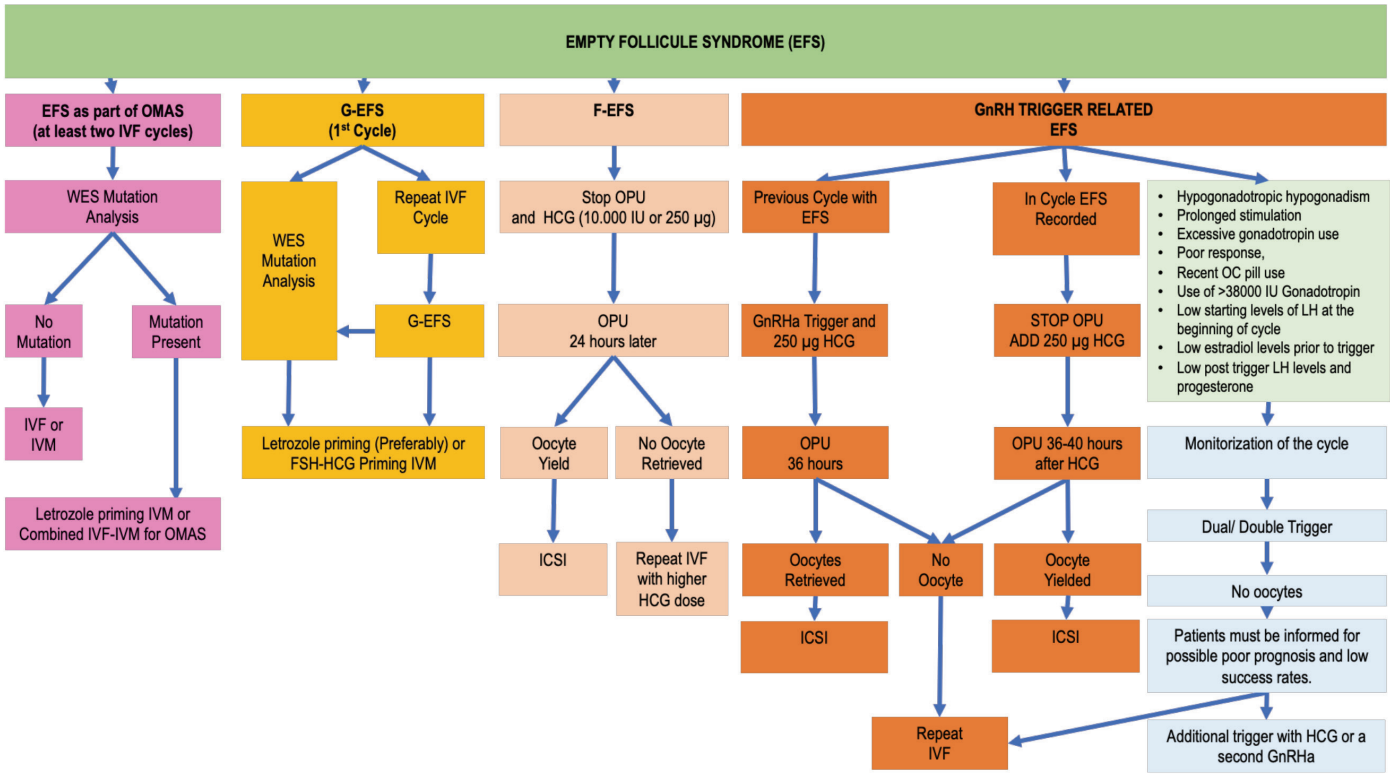

Figure 1. Empty follicule syndrome

EFS: Empty follicle syndrome, OMAS: Oocyte maturation abnormalities, IVF: In vitro fertilization, G-EFS: Genuine-EFS, F-EFS: False-EFS, OPU: Oocyte pick up, WES: Whole exome sequencing, IVM: In vitro maturation, FSH: Follicle-stimulating hormone, LH: Luteinizing hormone, HCG: Human chorionic gonadotropin, ICSI: Intracytoplasmic sperm injection, GnRHa: Gonadotrophin-releasing hormone analogues, OC: Oral contraceptive

Ethics Committee Approval: This study was approved by the Clinical Research Ethics Committee of Samsun University, Turkey (decision date: 26.04.2023, decision number: 2023/8/20).

Informed Consent: Written informed consent was obtained from the women studied.

Authorship Contributions

Surgical and Medical Practices: Ş.H., A.Baş., M.B., N.D.G., G.A., M.H.D., S.S.Ü., Concept: E.H., S.Ç., A.B., M.B., H.Ç., A.E.K., Design: J.T., S.L.T., Data Collection or Processing: C.S.Ç., M.C., Analysis or Interpretation: A.Baş., Literature Search: A.Baş., Writing: C.S.Ç.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

References

1. Coulam CB, Bustillo M, Schulman JD. Empty follicle syndrome. Fertil Steril. 1986;46:1153-5.
2. Revelli A, Carosso A, Grassi G, Gennarelli G, Canosa S, Benedetto C. Empty follicle syndrome revisited definition, incidence, etiology, early diagnosis and treatment. Reprod Biomed Online. 2017;35:132-8.
3. Aktas M, Beckers NG, Van Inzen WG, Verhoeff A, De Jong, D. Oocytes in the empty follicle: a controversial syndrome. Fertil Steril. 2005;84:1643-8.
4. Coskun S, Madan S, Bukhari I, Al-Hassan S, Al-Rejjal R, Awartani K. Poor prognosis in cycles following "genuine" empty follicle syndrome. Eur J Obstet Gynecol Reprod Biol. 2010;150:157-9.
5. Reichman DE, Greenwood E, Meyer L, Kligman I, Rosenwaks Z. Can in vitro fertilization cycles be salvaged by repeated administration of intramuscular human chorionic gonadotropin the day after failed injection? Fertil Sterile. 2012;98:671-4.
6. Mesen TB, Yu B, Richter KS, Widra E, Decherney AH, Segars JH. Prevalence of genuine empty follicle syndrome. Fertil Steril. 2011;96:1375-7.
7. Hourvitz A, Maman E, Brengauz M, Machtinger R, Dor J. In vitro maturation of patients with repeated in vitro fertilization failure due to "oocyte maturation abnormalities". Fertil Steril. 2010;94:496-501.
8. Sang Q, Zhou Z, Mu J, Wang L. Genetic factors as potential molecular markers of human oocyte and embryo quality. J Assist Reprod Genet. 2021;38:993-1002.
9. Zhou Z, Ni C, Wu L, Chen B, Xu Y, Zhang Z, et al. Novel mutations in ZP1, ZP2, and ZP3 cause female infertility due to abnormal zona pellucida formation. Hum Genet. 2019;138:327-37.
10. Altaf S, Bao J. Exome sequencing shines in empty follicle syndrome: zona pellucida gene mutations that manifest as genuine empty follicle syndrome. Fertil Steril. 2021;115:1170-1.
11. Zhang D, Zhu L, Liu Z, Ren X, Yang X, Li D, et al. A novel ZP3 mutation

causes empty follicle syndrome and abnormal zona pellucida formation. J Assist Reprod Genet. 2021;38:251-9.

12. Wassarman PM, Litscher ES. Zona Pellucida Genes and Proteins: Essential Players in Mammalian Oogenesis and Fertility. Genes (Basel). 2021;12:1266.
13. Li R, Albertini DF. Road to maturation: somatic cell interaction and self-organization of the mammalian oocyte. Nat Rev Mol Cell Biol. 2013;14:141-52.
14. Clarke HJ. Regulation of germ cell development via intercellular signaling in the mammalian ovarian follicle. Wiley Interdiscip Rev Dev Biol. 2018;7:10.1002/wdev.294.
15. Baena V, Terasaki M. Three-dimensional organization of transoral projections and other cytoplasmic extensions in the mouse ovarian follicle. Sci Rep. 2019;9:1262.
16. Wang Y, Lv C, Huang HL, Zeng MH, Yi DJ, Tan HJ, et al. Influence of mouse defective zona pellucida on granulosa cell apoptosis and developmental competence of oocytes. Biol Reprod. 2019;101:457-65.
17. Hatirnaz S, Hatirnaz E, Çelik S, Çalışkan CS, Tinelli A, Malvasi A, et al. Unraveling the Puzzle: Oocyte Maturation Abnormalities (OMAS). Diagnostics (Basel). 2022;12:2501.
18. Reichman DE, Hornstein MD, Jackson KV, Racowsky C. Empty follicle syndrome: Do repeat hCG administration really work? Fertil Steril. 2010;94:375-7.
19. Gonen Y, Balakier H, Powell W, Casper RF. Gonadotropin-releasing hormone agonist triggers follicular maturation for in vitro fertilization. J Clin Endocrinol Metab. 1990;71:918-22.
20. Itskovitz J, Boldes R, Levron J, Erlik Y, Kahana L, Brandes JM. Induction of preovulatory luteinizing hormone surge and prevention of ovarian hyperstimulation syndrome by gonadotropin-releasing hormone agonist. Fertil Steril. 1991;56:213-20.
21. Feferkorn I, Santos-Ribeiro S, Ubaldi FM, Velasco JG, Ata B, Blockeel C, et al. The HERA (Hyper-response Risk Assessment) Delphi consensus for the management of hyper-responders following in vitro fertilization. J Assist Reprod Genet. 2023;40:2681-95.
22. Tannus S, Turki R, Cohen Y, Son WY, Shavit T, Dahan MH. Reproductive outcomes after a single dose of gonadotropin-releasing hormone agonist compared with human chorionic gonadotropin for the induction of final oocyte maturation in hyperresponder women aged 35-40 years. Fertil Steril. 2017;107:1323-8.e2.
23. Castillo JC, Garcia-Velasco J, Humaidan P. Empty follicle syndrome after GnRHa vs. hCG triggering in COS. J Assist Reprod Genet. 2012;29:249-53.
24. Hatırnaz S, Hatırnaz E, Dahan MH, Tan SL, Ozer A, Kanat-Pektas M, et al. Is elective single-embryo transfer a viable treatment policy in in vitro maturation cycles? Fertil Steril. 2016;106:1691-5.
25. Hsu M, Mayer J, Aronshon M, Lanzendorf S, Muasher S, Kolm P, et al. Embryo implantation during in vitro fertilization and intracytoplasmic sperm injection: impact of cleavage status, morphology grade, and number of embryos transferred. Fertil Steril. 1999;72:679-85.
26. Neuber E, Mahutte NG, Arici A, Sakkas D. Sequential embryo assessment outperforms investigator-driven morphological assessment at selecting a good quality blastocyst. Fertil Steril. 2006;85:794-6.
27. Hatırnaz Ş, Hatırnaz ES, Ellibeş Kaya A, Hatırnaz K, Soyer Çalışkan C, Sezer H, et al. Oocyte maturation abnormalities: A systematic review of the evidence and mechanisms of this rare but difficult to manage fertility phenomena. Turk J Obstet Gynecol. 2022;19:60-80.
28. Hatirnaz S, Başbuğ A, Hatirnaz E, Tannus S, Hatirnaz K, Bakay K, et al. Can in vitro maturation overcome cycles of repeated oocyte maturation arrest? A classification system for maturation arrest and a cohort study. Int J Gynaecol Obstet. 2021;153:496-502.
29. Stevenson TL, Lashen H. Empty follicle syndrome: the reality of a controversial syndrome, a systematic review. Fertil Steril. 2008;90:691-8.
30. Kim JH, Jee BC. Empty follicle syndrome. Clin Exp Reprod Med. 2012;39:132-7.
31. Vutyavanich T, Piromlertamorn W, Ellis J. Immature oocytes in "apparent empty follicle syndrome": a case report. Case Rep Med. 2010;2010:367505.
32. Inan MS, Al-Hassan S, Ozand P, Coskun S. Transcriptional profiling of granulosa cells in a patient with recurrent empty follicle syndrome. Reprod Biomed Online. 2006;13:481-91.
33. Younis JS, Skournik A, Radin O, Haddad S, Bar-Ami S, Ben-Ami M. Poor oocyte retrieval is a manifestation of low ovarian reserve. Fertil Steril. 2005;83:504-7.
34. Onalan G, Pabuçcu R, Onalan R, Ceylaner S, Selam B. Empty follicle syndrome in two sisters after three cycles: case report. Hum Reprod. 2003;18:1864-7.
35. Baum M, Machtinger R, Yerushalmi GM, Maman E, Seidman DS, Dor J, et al. Recurrence of empty follicle syndrome with stimulated IVF cycles. Gynecol Endocrinol. 2012;28:293-5.
36. Yuan P, He Z, Zheng L, Wang W, Li Y, Zhao H, et al. Genetic evidence of a "genuine" empty follicle syndrome: a novel effective mutation in the LHCGR gene and review of the literature. Hum Reprod. 2017;32:944-53.
37. Xu Y, Wang E, Liu T, Wang S, Wu F, Zhao X, et al. Whole exome sequencing identifies a novel homozygous missense mutation of LHCGR gene in primary infertile women with empty follicle syndrome. J Obstet Gynaecol Res. 2023;49:2436-45.
38. Sun L, Fang X, Chen Z, Zhang H, Zhang Z, Zhou P, et al. Compound heterozygous ZP1 mutations cause empty follicle syndrome in infertile sisters. Hum Mutat. 2019;40:2001-6.
39. Pujalte M, Camo M, Celton N, Attencourt C, Lefranc E, Jedraszak G, et al. ZP1 gene mutation in a patient with empty follicle syndrome: A case report and literature review. Eur J Obstet Gynecol Reprod Biol. 2023;280:193-7.
40. Okutman O, Tarabeux J, Muller J, Viville S. Evaluation of a Custom-design Gene Panel as a Diagnostic Tool for Human Non-Syndromic Infertility. Genes (Basel). 2021;12.
41. Chen T, Bian Y, Liu X, Zhao S, Wu K, Yan L, et al. Recurrent Missense Mutation in ZP3 Causes Empty Follicle Syndrome and Female Infertility. Am J Hum Genet. 2017;101:459-65.
42. Kong N, Xu Q, Shen X, Zhu X, Cao G. Case report: A novel homozygous variant of ZP3 is associated with empty follicle syndrome. Front Genet. 2023;14:1256549.